

AD _____

Award Number: DAMD17-99-1-9340

TITLE: Development of an erbB Antagonist

PRINCIPAL INVESTIGATOR: Amanda Simcox, Ph.D.

CONTRACTING ORGANIZATION: The Ohio State University
Research Foundation
Columbus, Ohio 43210-1063

REPORT DATE: July 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

| REPORT DOCUMENTATION PAGE | | | Form Approved OMB No. 074-0188 | |
|---|---|---|--|--|
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503 | | | | |
| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE July 2000 | 3. REPORT TYPE AND DATES COVERED Annual (1 Jul 99 - 30 Jun 00) | | |
| 4. TITLE AND SUBTITLE Development of an erbB Antagonist | | 5. FUNDING NUMBERS DAMD17-99-1-9340 | | |
| 6. AUTHOR(S) Amanda Simcox, Ph.D. | | | | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Ohio State University Research Foundation Columbus, Ohio 43210-1063 E-MAIL: simcox.1@osu.edu | | 8. PERFORMING ORGANIZATION REPORT NUMBER | | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012 | | 10. SPONSORING / MONITORING AGENCY REPORT NUMBER | | |
| 11. SUPPLEMENTARY NOTES | | | | |
| 12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited | | | 12b. DISTRIBUTION CODE | |
| 13. ABSTRACT (Maximum 200 Words) <p>Receptor tyrosine kinases of the erbB family play pivotal roles in growth and differentiation and aberrant activation of these receptors is associated with human cancers. In particular, ErbB-2 dysfunction has been linked to about 30% of breast cancers with poor prognosis. Correspondingly, great efforts are being made to develop therapies that target ErbB pathways. ErbB-2 is activated by the neuregulins in heterodimers with the neuregulin receptors ErbB-3 and ErbB-4. An antagonistic neuregulin that down regulates ErbB signaling could function as an anti-tumor agent with significant clinical potential. The purpose here is to develop such a factor. In previous work, the <i>Drosophila</i> system was used to demonstrate that an antagonistic neuregulin-like factor could be made by deleting the EGF domain or by insertion of the EGF domain from a natural inhibitor. In this project, a vertebrate neuregulin with an EGF domain deletion and a factor with the EGF domain from the inhibitor were made and appear to have inhibitory activity <i>in vitro</i>. The activity of the factors is now being tested against human breast-cancer cell lines. Corresponding constructs have been made to generate transgenic mice and these will provide an <i>in vivo</i> test of the factors.</p> | | | | |
| 14. SUBJECT TERMS Breast Cancer | | | 15. NUMBER OF PAGES 13 | |
| | | | 16. PRICE CODE | |
| 17. SECURITY CLASSIFICATION OF REPORT Unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified | 20. LIMITATION OF ABSTRACT Unlimited | |

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Amada Sumay 7/25/00

Table of Contents

| | |
|--|-------------|
| Cover..... | 1 |
| SF 298..... | 2 |
| Foreword..... | 3 |
| Table of Contents..... | 4 |
| Introduction..... | 5 |
| Body..... | 5 |
| Key Research Accomplishments..... | 7 |
| Reportable Outcomes..... | 7 |
| Conclusions..... | 7 |
| References..... | 7 |
| Appendices..... | 7-13 |

(5) Introduction

Receptor tyrosine kinases (RTKs) of the erbB family play pivotal roles in growth and differentiation during normal development and aberrant activation of these receptors is associated with a significant number and variety of human cancers. In particular, erbB-2 dysfunction has been linked to about 30% of breast cancers and these have a poor prognosis. Correspondingly, great efforts are being made to develop therapies that target erbB pathways. The purpose of this work is to develop a vertebrate neuregulin antagonist that has potential use as an anti-tumor agent in some breast cancers that involve erbB-2 dysfunction. The rationale for the proposed work is based on a novel finding in the fly system which shows deletion of the EGF domain, or insertion of the EGF domain from a natural inhibitor, converts the fly neuregulin, Vein, into an antagonist [1](and unpublished data). Similar modifications are being made in a vertebrate neuregulin and the ability of the factors to act as inhibitors will be tested *in vitro* and *in vivo*.

(6) Body

Three tasks are described in the approved statement of work and our progress on the first two tasks, which are part of the first year of the program, are described below.

Task 1. To test the function of NRG-1: ΔEGF in cell culture.

- Creation of an EGF deletion in NRG-1B (months 1-2)
- Testing effect of NRG-1: ΔEGF on NRG-1-activation of erbB receptors in mouse cells (months 2-10)
- Testing effect of NRG-1: ΔEGF on human breast cancer cell lines (months 10-26)

Creation of an EGF deletion in NRG-1B

Our work in the fly system showed that deletion of the EGF domain from the fly neuregulin converted it into an erbB antagonist (unpublished data). The goal here was to create a similar change in a vertebrate neuregulin. The NRG-1ΔEGF construct was made according to the scheme is shown in Figure 1. We decided to also make a construct that has the EGF domain from the fly inhibitor called Argos (Aos) as our work in the fly system suggested this may be a more powerful inhibitor than the EGF deletion factor [1](and unpublished data). The scheme to create this construct (NRG-1::Aos-EGF) is also shown in Figure 1.

Testing effect of NRG-1: ΔEGF on NRG-1-activation of erbB receptors in mouse cells

We made large preparations of DNA corresponding to the two factors (NRG-1ΔEGF and NRG-1::Aos-EGF) and sent these to our collaborator Dr. Stern at Yale medical school. Dr. Stern transfected COS-7 cells with the DNA constructs and produced conditioned media containing the encoded secreted factors. The activity of the factors was assayed in tissue culture cells expressing the receptor ErbB4. In this experiment the ability of the mutant factors to inhibit

activation by native neuregulin was tested. Mouse cells expressing ErbB4 were treated with neuregulin or neuregulin in conjunction with a mutant factor. The receptor was immunoprecipitated and analyzed by western blotting with an anti-phosphotyrosine antibody. The level of phosphorylation is a measure of receptor activation. The results showed that both factors were able to block activation of the receptor by neuregulin (Fig. 2). There was some variation between factor preparations but positive results were obtained from 3/4 of the preparations. Some variation is to be expected given that the efficiency of transfection differs between experiments and the concentration of the factor in any given preparation was not determined. In sum these results are very encouraging.

Testing effect of NRG-1: Δ EGF on human breast cancer cell lines

We have obtained and set up in culture six human breast-cancer cell lines (MDA-MB-175, MDA-MB-468, BT-20, MCF7, T47D, and MDA-MB-453). We will test the effect of NRG-1 Δ EGF and NRG-1::Aos-EGF on these cancer cells. The level of ErbB phosphorylation and growth assays will be performed on controls and cells treated with the factors. This work has been initiated but we have no results to date.

Task 2. To generate and analyze the phenotypes of transgenic mice which express NRG-1: Δ EGF in heart and breast.

- Creation of NRG-1: Δ EGF transgene for expression in early embryos (months 10-11)
- Generation and phenotypic analysis of NRG-1: Δ EGF transgenic mice (months 11-24)
- Creation of NRG-1: Δ EGF transgene for expression in breast (months 15-16)
- Generation and phenotypic analysis of MMTV NRG-1: Δ EGF transgenic mice (months 16-32)

Creation of NRG-1: Δ EGF transgene for expression in early embryos

We proposed to test the ability of the mutant neuregulins to function as dominant negative proteins that mimic loss of function phenotypes seen in neuregulin knockout mice. To do this we had to first make a construct suitable for generating transgenic mice that express the gene in the heart. This turned out to be a much more complex construction than was anticipated. However, we have been successful and have in fact generated two such constructs, for the reasons stated above we made an additional construct with the EGF domain from Argos. The scheme depicting the construction is shown in Figure 3.

Generation and phenotypic analysis of NRG-1: Δ EGF transgenic mice

Large-scale preparations of pMHC NDF Δ EGF polyA and pMHC NDF:AOS polyA were made and digested to release the gene fragment (Fig. 4). The digests were run on a gel and the correct gel slices were delivered to the mouse facility. The injections will be performed in August 2000.

(7) Key Research Accomplishments

- Generation of constructs for *in vitro* and *in vivo* testing of mutant neuregulins
- Demonstration that NRG-1 Δ EGF and NRG-1::Aos-EGF can function as competitive inhibitors of native neuregulin.

(8) Reportable Outcomes

- Research opportunity for a graduate student (Jonathan Butchar)

(9) Conclusions

This has been a successful first year and we have accomplished the tasks as set out in the statement of work. Two lengthy DNA constructions have been successfully completed (Figs. 1&3) and the first test of the mutant factors produced very encouraging results (Fig. 2). Given these accomplishments we will continue with the tasks as described in the original proposal. In the near future we will have a clearer idea as to whether the mutant neuregulins function in the whole animal and whether the inhibitory effects seen in mouse cells also translate to human breast cancer cells.

(10) References

1. Schnepf, B., *et al.*, *EGF domain swap converts a Drosophila EGF-receptor activator into an inhibitor*. *Genes & Dev.*, 1998. **12**: p. 908-913.

(11) Appendices

Figures 1-4

Tissue Culture constructs

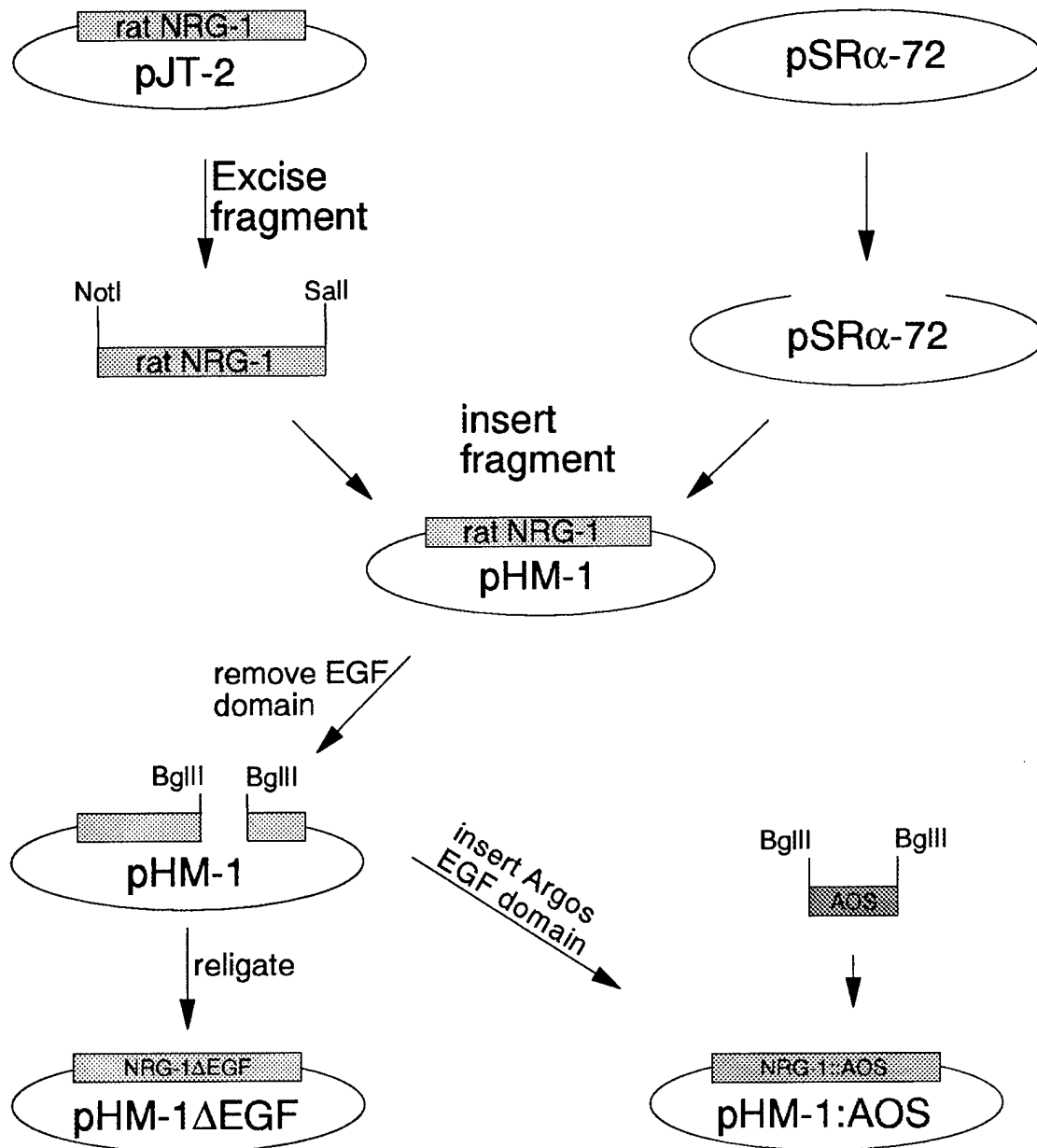


Figure 1. Creation of the pHM-1 constructs. Rat NRG-1 was excised from pJT-2 with a *NotI* / *Sall* digest and inserted into pSRα72 to create pHM-1. The NRG-1ΔEGF fragment was created by excising the EGF domain via a *BglII* digest. The NRG-1::AOS fragment was created by inserting the *Drosophila* Argos EGF domain into the *BglII* site.

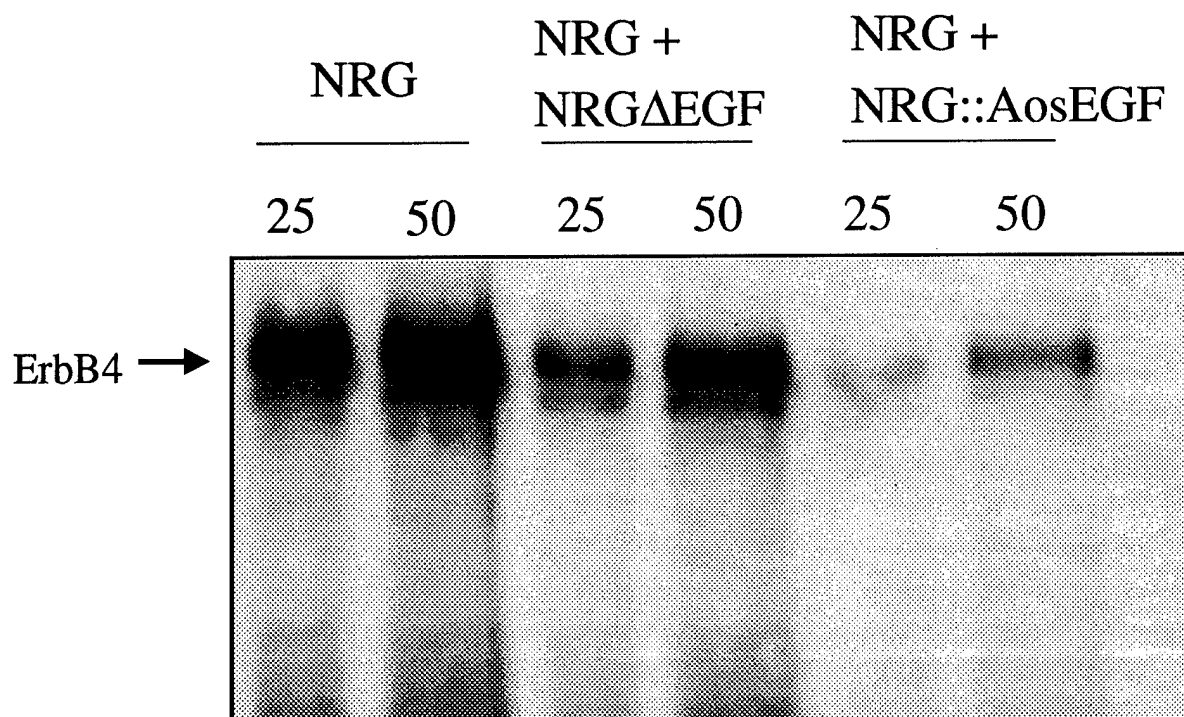


Figure 2. NRG-1_EGF and NRG-1::Aos-EGF inhibit the ability of native neuregulin to induce phosphorylation of ErbB4. Mouse cells expressing ErbB4 were exposed to neuregulin (NRG) or neuregulin in combination with the mutant factors NRG-1_EGF (NRG + NRG_EGF) or NRG-1::Aos (NRG + NRG::AosEGF). The volume of conditioned medium containing neuregulin added to the cells was either 25ul or 50 ul. The volume of conditioned medium with mutant factors was kept constant. ErbB4 was immunoprecipitated subjected to SDS-PAGE followed western blotting with anti-phosphotyrosine. The arrow indicates the position of ErbB4. The intensity of the band indicates the degree of phosphorylation. The intensity of the bands seen after stimulation with neuregulin (NRG) was reduced by co-incubation with conditioned media containing the mutant factors. In this experiment NRG-1::Aos-EGF appeared to be a more potent inhibitor.

Transgenic mouse constructs.

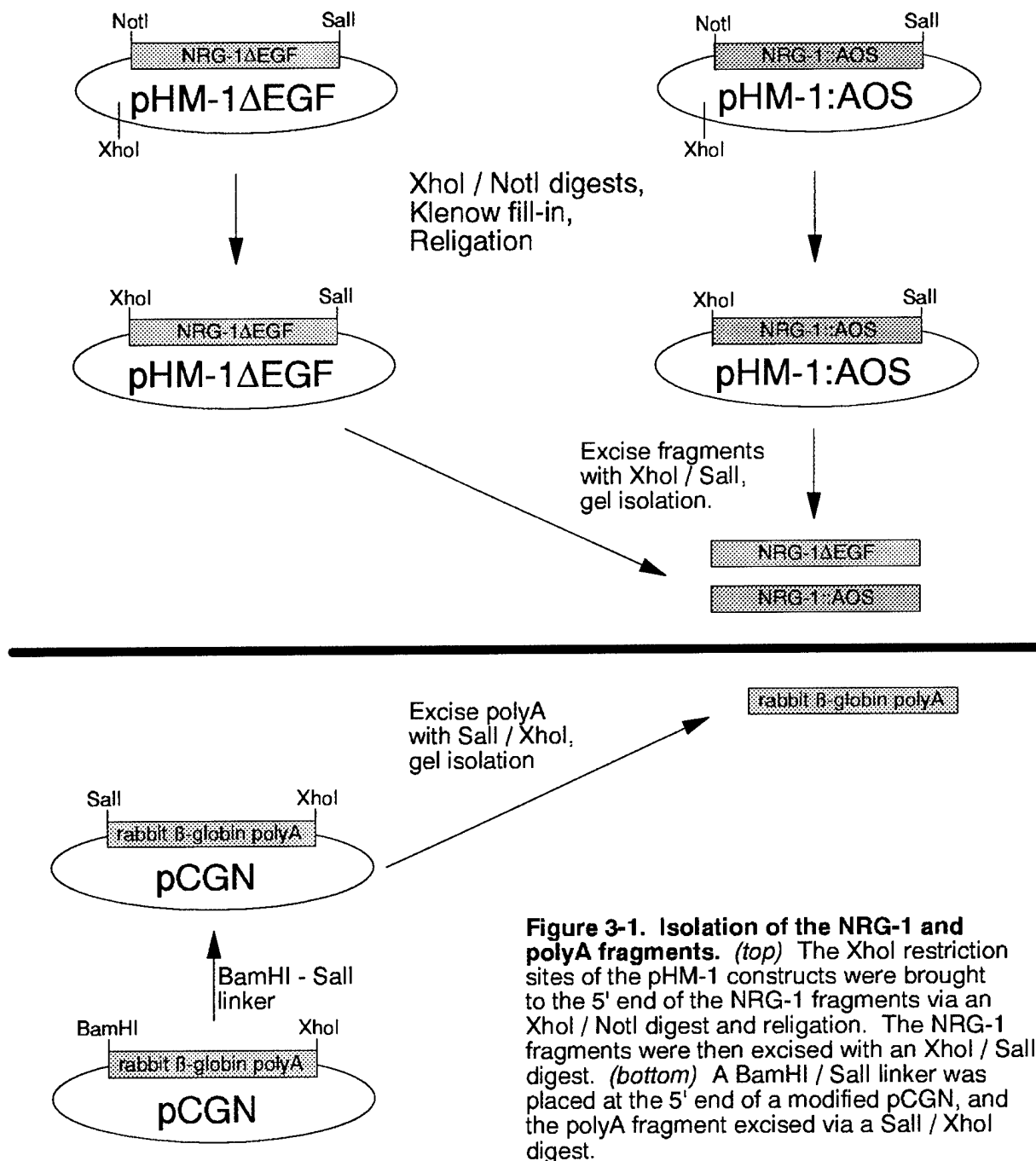


Figure 3-1. Isolation of the NRG-1 and polyA fragments. (top) The XhoI restriction sites of the pHM-1 constructs were brought to the 5' end of the NRG-1 fragments via an XhoI / NotI digest and religation. The NRG-1 fragments were then excised with an XhoI / Sall digest. (bottom) A BamHI / Sall linker was placed at the 5' end of a modified pCGN, and the polyA fragment excised via a Sall / XhoI digest.

Transgenic mouse constructs, cont'd.

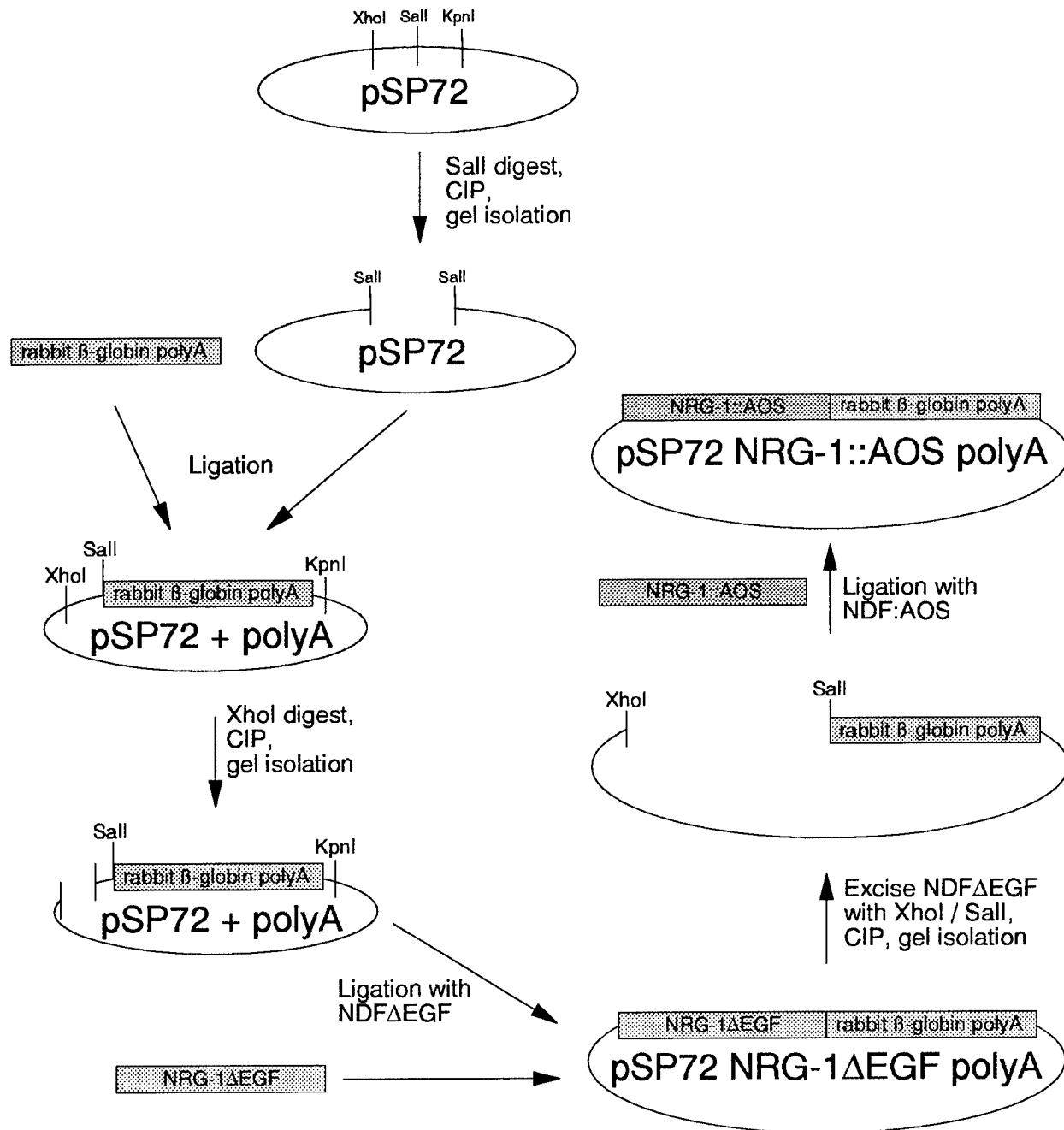


Figure 3-2. Creation of the intermediate mouse constructs. The polyA fragment excised from pCGN was inserted into the **Sall** site of pSP72, and the NRG-1ΔEGF fragment was then placed into the **XhoI** site. An **XhoI / Sall** digest was performed to excise the NRG-1ΔEGF fragment and the NRG-1::AOS fragment was then inserted.

Transgenic mouse constructs, cont'd.

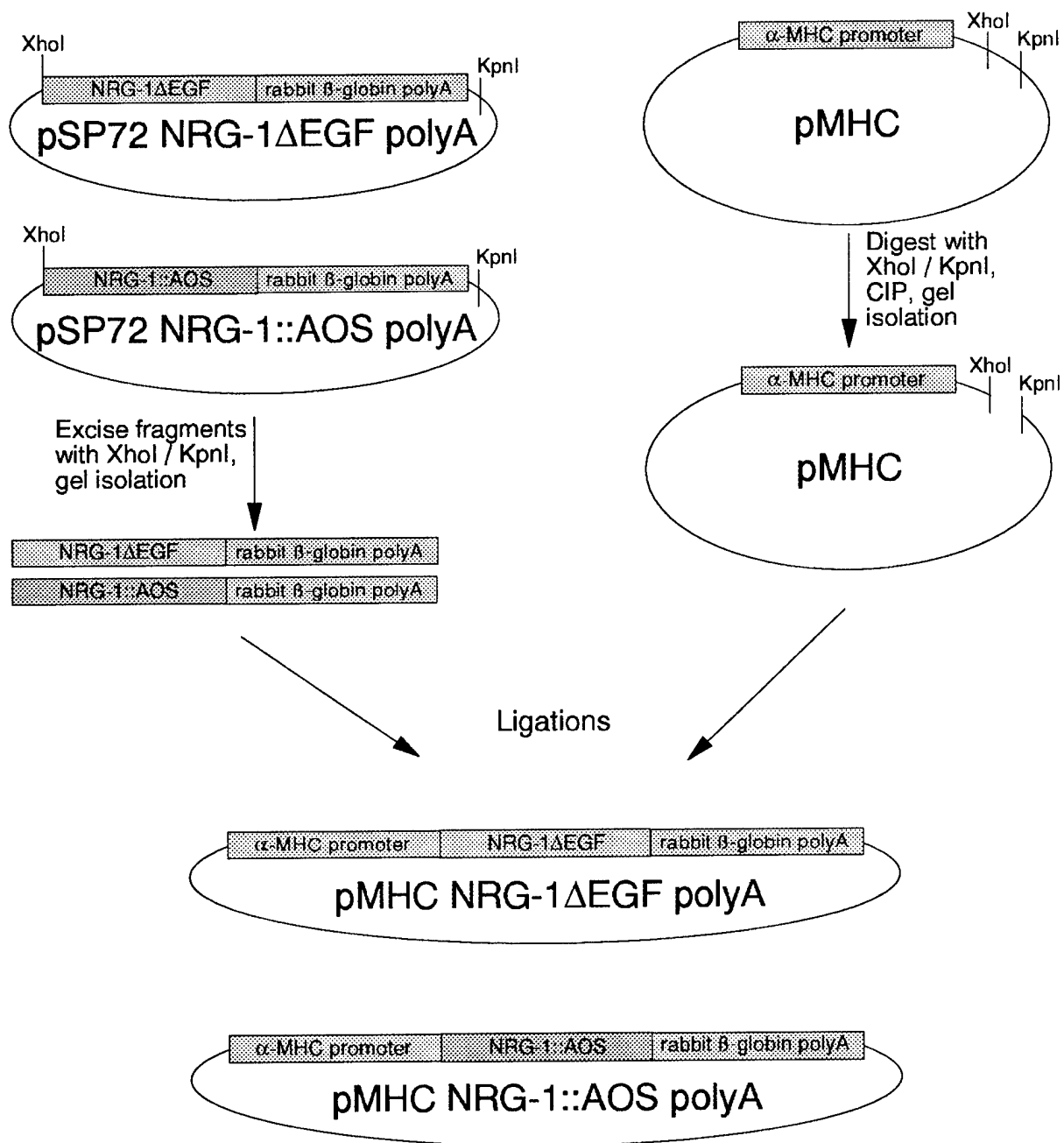


Figure 3-3. Creation of the final mouse constructs. The NRG-1 / polyA fragments were excised with *XhoI* / *KpnI* digests and placed at the 3' end of the pMHC α -MHC promoter.

DNA Preparation for mouse injections.

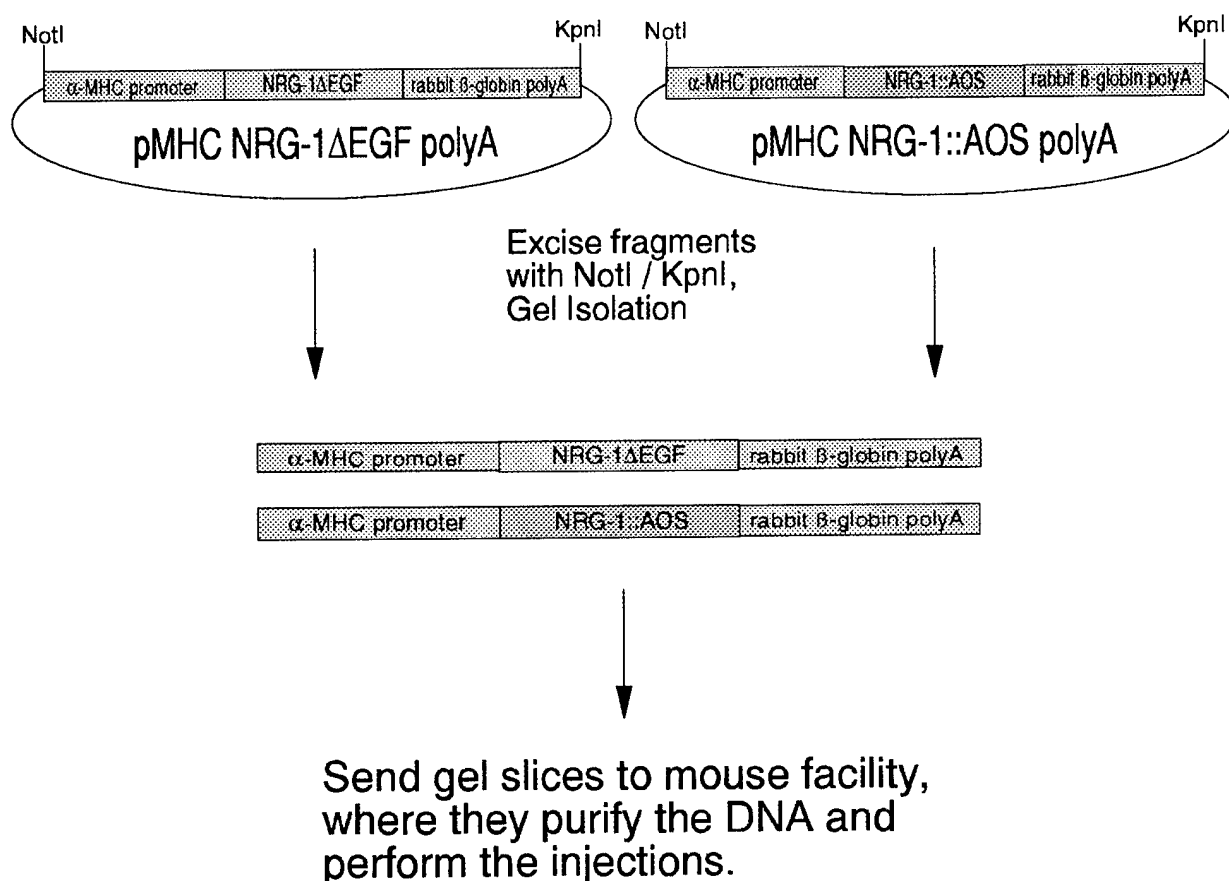


Figure 4. Preparation of DNA for mouse injections. The fragments were excised via NotI / KpnI digests and purified on a 0.6% agarose gel. Slices containing the target fragments were removed, weighed, and sent to the transgenic mouse facility.